

Regulated expression of the *Leishmania major* surface virulence factor lipophosphoglycan using conditionally destabilized fusion proteins

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Surface glycoconjugates play important roles in the infectious cycle of *Leishmania major*, including the abundant lipophosphoglycan (LPG) implicated in parasite survival in the sand fly vector and the initial stages of establishment in the mammalian host macrophage. We describe a system for inducible expression of LPG, applying a novel protein-based system that allows controlled degradation of a key LPG biosynthetic enzyme, UDP-galactopyranose mutase (UGM). This methodology relies on a mutated FK506-binding protein (FKBP) destabilizing domain (dd) fused to the protein of interest; in the absence of rapamycin analogs, such as Shld1, the dd domain is destabilized, leading to proteasomal degradation, whereas drug treatment confers stabilization. Tests in *L. major* using dd fusions to a panel of reporters and cellular proteins confirmed its functionality, with a high degree of regulation and low background, and we established the kinetics of protein activation and/or loss. Two inexpensive and widely available ligands, FK506 and rapamycin, functioned similarly to Shld1, without effect on *Leishmania* growth or differentiation. We generated parasites lacking UGM through deletion of the *GLF* gene and substitution with a *ddGLF* fusion construct, either as chromosomal knockins or through episomal complementation; these showed little or no LPG expression in the absence of inducer, whereas in its presence, high levels of LPG were attained rapidly. Complement lysis tests confirmed the correct integrity of the *Leishmania* LPG coat. These data suggest that the dd approach has great promise in the study of LPG and other pathways relevant to parasite survival and virulence.

inducible expression | FK506 | glycoconjugates | pathogen | trypanosomatid protozoa

Leishmaniasis is a parasitic disease infecting more than 12 million people worldwide and constituting a significant public health burden in affected areas (1). It is caused by protozoan parasites of the genus *Leishmania* that, depending on the species, cause a range of pathologies, from cutaneous or mucocutaneous to the fatal visceral leishmaniasis. Since the emergence of reverse genetic approaches, including homologous gene replacement and heterologous expression, our ability to probe gene function and expression related to parasite virulence has advanced considerably (2, 3). Nonetheless, the *Leishmania* toolkit would benefit from further development incorporating methods better able to cope with challenges arising from the diploid nature of the *Leishmania* genome, as well as research into the limitations of current regulatable systems, which are typically based on various strategies affecting transcriptional initiation (4). These face some challenges because of the unique aspects of transcription and gene expression in trypanosomatid protozoans (5, 6).

Protein-based regulatable systems offer some potential for overcoming limitations arising from regulatable transcription systems. Recently, Wandless and colleagues described a system in which protein levels are controlled through regulated degradation of a *cis*-acting domain joined to the target protein of interest (7). The destabilizing protein domain consists of a modified, 108-aa FK506/rapamycin-binding protein (FKBP) engineered to bind selectively to the nontoxic FK506/rapamycin analog, Shld1. In the presence of

Shld1, the FKBP destabilization domain (dd) is properly folded, thereby conferring stability to the dd fusion protein, whereas in its absence, destabilization of the dd structure targets the protein for degradation. This method was applied recently to the Apicomplexan parasites *Plasmodium falciparum* and *Toxoplasma gondii* (8, 9), and here we report success with 2 species of *Leishmania* parasites. Of practical importance, the relative insensitivity of *Leishmania* to FKBP ligands, including rapamycin and FK506, allows them to be used in vitro, advantageous because of their lower cost and widespread availability.

We applied this to the development of regulated expression of a key *Leishmania* virulence molecule, lipophosphoglycan (LPG). The cell surface of *Leishmania* is densely covered with a variety of glycoconjugates playing major roles throughout the parasite's life cycle (10). One of the most intensely studied is the abundant LPG, consisting of a long phosphoglycan chain of galactose-mannose-phosphate-based repeating units attached to the plasma membrane through a GPI anchor (11). In *L. major*, LPG plays a role in the establishment of the infection of promastigotes in the sand fly vector, acting as an adhesin that promotes attachment of the parasites to the insect midgut wall through binding to a galectin receptor (12). After inoculation of the parasite into the mammalian host by the sand fly bite, LPG plays important roles in the establishment of the infection by conferring resistance to lysis by complement, protection from oxidative damage, and remodeling of the initial phagolysosome, including the induction of a transient delay in phagolysosomal fusion (13–15). The roles of LPG deduced by a variety of biochemical and cellular studies have been supported by studies of mutant parasites lacking genes of the LPG biosynthetic pathway (13, 16–19).

LPG is assembled through a series of steps involving a diverse set of proteins located in cellular compartments, including the cytosol, glycosome, and secretory pathways (11, 20). A key linkage within the LPG core involves galactosylfuranose (Galf), an unusual parasite sugar not made by the mammalian host (21, 22). The precursor for Galf is UDP-Galf, which arises from the action of cytosolic UDP-galactopyranose mutase (UGM, encoded by the gene *GLF*) (16, 23), and *glf*[−] knockouts confirm the requirement for this gene in *Leishmania* LPG biosynthesis (18). Although Galf occurs on other *Leishmania* glycoconjugates, including the abundant glycosylinositolphosphatidylinositols (GIPLs), previous studies establish that GIPLs play little role in *L. major* infectivity (24) and, accordingly, *glf*[−] knockouts show phenotypes identical to *lpg1*[−] parasites

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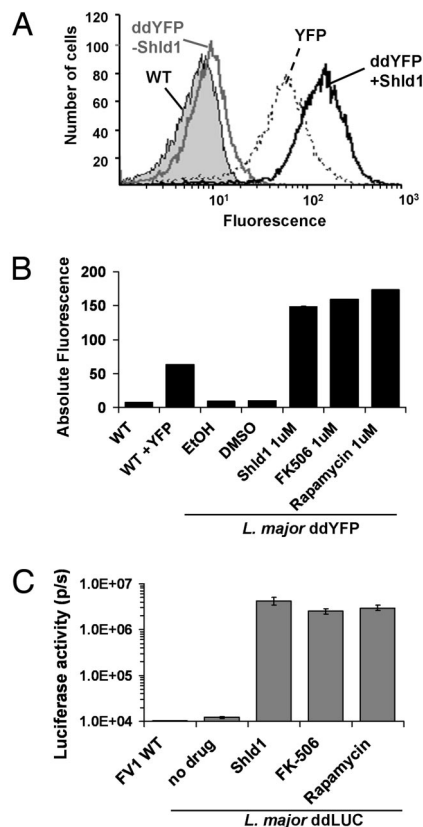


Fig. 1. Functionality of the FKBP dd system in *L. major*. WT, YFP-expressing, ddYFP-expressing, or ddLUC-expressing promastigotes were grown in the presence or absence of $1 \mu\text{M}$ Shd1, FK506, and rapamycin for 24 h, and fluorescence levels were measured by flow cytometry (A and B) or luciferase activity assay (C). (A) Histogram plot representative of the distribution of fluorescence levels in the different populations of cells. (B) Average mean fluorescence \pm SEM relative to the maximum fluorescence intensity measured in the series. Experiments were performed in triplicate. (C) Average luciferase activity \pm SEM (p/s) for 3 independent clones. All transfectants shown express proteins from transgenes inserted into the rRNA locus (*SSU::IR1PHLEO-YFP*, *-ddYFP*, or *-LUC*).

lacking LPG alone (18, 19, 25). In this work, we used the dd fusion approach to regulate LPG expression through control of UGM levels, yielding parasites that when placed in the “on” and “off” states closely resemble WT and LPG-deficient mutants.

Results

The Modified FKBP dd Is Functional in *L. major*. Preliminary tests suggested that the stabilizing ligand Shd1 had little toxicity to *L. major* at 1 μ M. We expressed a dd yellow fluorescent protein (ddYFP) in *L. major* after integration of constructs into the SSU ribosomal locus (*SSU::IR1PHLEO-ddYFP*) and compared it to YFP expression in similar independent transfectants (*SSU::IR1PHLEO-YFP*). Parasites expressing unmodified YFP were highly fluorescent [62 fluorescence units (FU)], whereas those expressing ddYFP were only slightly more fluorescent than untransfected controls (9 vs. 7 FU; Fig. 1 *A* and *B*). Notably, addition of 1 μ M Shd1 to the ddYFP-expressing *Leishmania* for 24 h yielded strongly fluorescent parasites (\approx 150 FU), 16-fold greater than seen in the absence of ligand (62-fold after correcting for background fluorescence; Fig. 1 *A* and *B*). Similar results were obtained with ddYFP expressed from multicopy episomal vectors.

We tested a cytosolic dd-Luciferase (ddLUC) construct after expression in *L. major*. In the absence of Shld1, ddLUC transfectants showed minimal luminescence, close to the WT background (1.0×10^4 vs. 1.2×10^4 photons per second; Fig. 1C). After

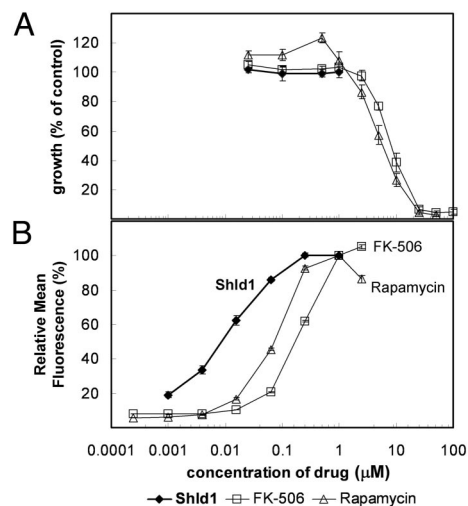


Fig. 2. Effects of Shld1 alternative ligands, FK506, and rapamycin on *L. major* growth, ddYFP induction, and metacyclogenesis in *L. major*. Promastigotes (*SSU::IR1PHleo- Δ ddYFP*) were treated with different concentrations of drug or solvent (negative control) for 24 h, and cell density (A) and ddYFP fluorescence (B) were then measured by Coulter and flow cytometry, respectively. Error bars represent SEM of 2 independent experiments performed in duplicate.

incubation in the presence of 1 μ M Shld1 for 24 h, LUC activity rose 350-fold (4.3×10^6 photons per second; Fig. 1C).

FK506 and Rapamycin Can Be Used as ddFKBP Stabilizing Compounds in *L. major*. Shld1 was developed as a nontoxic analog of the FKBP ligands FK506 or rapamycin, which show toxicity or deleterious effects on eukaryotic cells (26–28). Although the *Leishmania sp.* genomes reveal several TOR kinase homologs (<http://tritrypdb.org/tritrypdb/>), the action of these compounds on *Leishmania* parasites has received less attention (29). We established the growth inhibition IC₅₀ for Shld1, FK506 or rapamycin as 8.4 ± 0.3 , 7.7 ± 0.2 , or 4.9 ± 0.5 μ M, respectively (Fig. 2A, Table S1). We then tested ddYFP expression after growth of ddYFP-expressing *L. major* for 24 h in the presence of 1 μ M ligand (Fig. 1B). FK506 and rapamycin induced strong YFP fluorescence in ddYFP-expressing *L. major*, slightly higher than seen with Shld1 (Fig. 1B). Similarly, FK506 and rapamycin induced high levels of LUC expression in ddLUC expressing *L. major*—200-fold and 250-fold that seen in the absence of ligand (Fig. 1C).

Dose-response curves were performed for all 3 compounds by using the ddYFP-expressing *L. major* and 24 h of induction (Fig. 2B). Shld1 was the most potent, with an effective concentration (EC₅₀) of 10 nM, followed by rapamycin (60 nM) and FK506 (200 nM). As seen in previous studies, ddYFP expression was “tunable,” in that variation in ligand concentration yielded variation in the average YFP fluorescence homogeneously within the cell population (Fig. S1A). The concentration required for maximal ddYFP expression was 250 nM for Shld1 and 1 μM for FK506 and rapamycin (Fig. 2B). At 1 μM ligand, neither parasite growth nor differentiation to the infective metacyclic form was affected (Fig. 2A and Fig. S1B).

Kinetics of ddFKBP-Controlled Induction and Loss in *L. major*. Early log-phase promastigotes were treated with different concentrations of stabilizing compounds, and ddYFP expression was assessed by flow cytometry for 24 h. Maximum ddYFP protein levels were achieved within 8 h for all 3 drugs, with similar kinetics (Fig. 3A–C).

To study decay, parasites were incubated 24 h with ligand, and then were washed once and inoculated into fresh media. More than 2 h after removal of Shd1 and FK506, ddYFP expression had decreased to nearly basal levels, with a half-life of about 30 min (Fig.

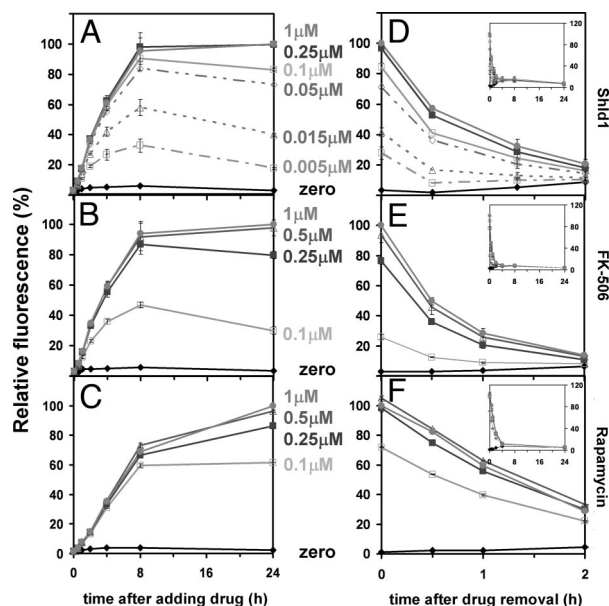


Fig. 3. Characterization of the kinetics of expression and loss of ddYFP fluorescence upon addition or removal of Shld1, FK506, or rapamycin. (A–C) Drugs were added to logarithmic-phase cultures of *L. major* promastigotes (*SSU::IR1PHLEO-ddYFP*), and ddYFP fluorescence levels were measured by flow cytometry. (D–F) After 24 h in the presence of ligands, parasites were washed and then suspended in fresh culture medium lacking drugs, and fluorescence levels were measured periodically. (A and D) Shld1. (B and E) FK506. (C and F) Rapamycin. D–F Insets show the experiment extended to 24 hr. Data are presented as the average mean fluorescence \pm SEM relative to the maximum fluorescence intensity measured in the series. Two or 3 independent experiments were performed in triplicate.

3 *D–F*). The decay of ddYFP expression was slower after rapamycin treatment, reaching basal levels after more than 4 h and with a decay half-life of about 75 min (Fig. 3 *D* and *E*). Western blot analysis with anti-GFP antibody, which also recognizes YFP, confirmed these findings (Fig. S2). As summarized in Table S1, when used at 1 μ M, FK506 and rapamycin can be used effectively as FKBP dd stabilizing ligands in *Leishmania*.

Application of the ddFKBP System to *Leishmania* Proteins and in *Leishmania braziliensis*. We generated dd fusions for a variety of *L. major* proteins, including DHCH1 (5,10-methylenetetrahydrofolate dehydrogenase/5,10-methenyltetrahydrofolate cyclohydrolase), FTL (formate-tetrahydrofolate ligase), and DHFR-TS (dihydrofolate reductase thymidylate-synthase). Each fusion protein was expressed in *L. major*, and its levels were determined by Western blotting after 24 h in the presence or absence of inducer. For each protein, strong regulation was seen in orders of magnitude similar to those seen for the reporter proteins. In the absence of inducer, expression of the fusion protein was negligible at the conditions tested (Fig. S3A). Similar results were obtained with ddYFP, ddLUC, ddFTL, or ddDHCH1 proteins expressed in promastigotes of *L. braziliensis*, an early-diverging species belonging to the subgenus *Viannia* (Fig. S3B and C). These results suggest that the ddFKBP fusion approach will have broad applicability in *Leishmania*.

Generation of an *L. major* Mutant Lacking LPG Through Ablation of *Galf* Synthesis. We generated a *gltf*⁻ null mutant (formally, *gltfΔHYG/gltfΔPAC*) by standard techniques of gene replacement (Fig. 4 and Fig. S4A), which requires 2 successive rounds of targeting because *Leishmania* is predominantly diploid (30). Generation of the correct replacement and loss of *GLF* were confirmed by PCR (Fig. S4B). Western blotting with the monoclonal antibody

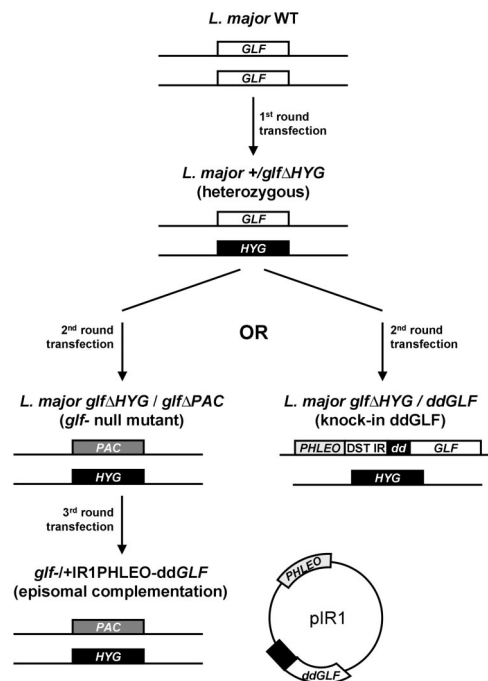


Fig. 4. Generation of *glf⁻* mutants expressing ddUGM from multicopy episomes or chromosomal knockins. *L. major* LV39c15 parasites were transfected with a construct replacing the *GLF* ORF of the first allele with that encoding a hygromycin B resistance marker (*HYG*). This heterozygous line (+/*glf*Δ*HYG*) was used in 2 ways. As depicted on the left branch, the remaining *GLF* allele was replaced with that encoding a puromycin resistance marker (*PAC*), yielding a homozygous *glf⁻* null mutant (formally, *glf*Δ*HYG*/*glf*Δ*PAC*). *GLF* was then restored by transfection with pIR1PHLEO-dd*GLF*, yielding *glf⁻*/pIR1PHLEO-dd*GLF* parasites. Alternatively, as depicted in the right branch, the remaining *GLF* allele in +/*glf*Δ*HYG* was replaced by transfection with a PHLEO-dd*GLF* knockin-targeting construct, yielding *glf*Δ*HYG*/dd*GLF*.

WIC79.3, which recognizes the galactose-containing side chains of LPG and other phosphoglycans, showed that the *glf*⁻ parasites lacked LPG (Fig. S4C) but retained expression of the high-molecular weight proteophosphoglycans (PPGs), which lack Galf (20). In contrast, control mutant *lpg2*⁻, lacking the Golgi GDP-mannose transporter required for the synthesis of all phosphoglycans, showed loss of both LPG and PPG (Fig. S4C). These findings agree with previous work using another *L. major glf*⁻ strain (18).

Complementation of *L. major glf⁻* with ddUGM Confers Regulatable LPG Expression. A ddUGM fusion protein was expressed in the *glf⁻* mutant by transfection with a multicopy episomal vector, pIR1PHLEO-*ddGLF* (Fig. 4). *glf⁻*/+pIR1PHLEO-*ddGLF* transfectants were grown in the presence or absence of 1 μ M FK506 for 24 h, and the levels of ddUGM protein were assessed by Western blotting. Some variations in basal LPG and/or ddUGM induction were seen in these transfectants that were attributed to differences in copy number of the episomal vector (Fig. S5).

Dose-response studies for several clonal lines showing minimal basal LPG and ddUGM expression were performed (one is shown in Fig. 5A). In the absence of Shld1, a low level of LPG expression was found, whereas addition of just 10 nM Shld1 resulted in strong LPG expression without detectable expression of ddUGM protein, suggesting that very low protein levels are required for LPG synthesis. WT levels of LPG were attained at just 50 nM Shld1 (Fig. 5A).

Knockin Approaches Yield Improvements in *ddGLF*-Dependent LPG Down-Regulation. The utility of regulatable systems is ultimately dependent on their ability to cross biologically relevant thresholds

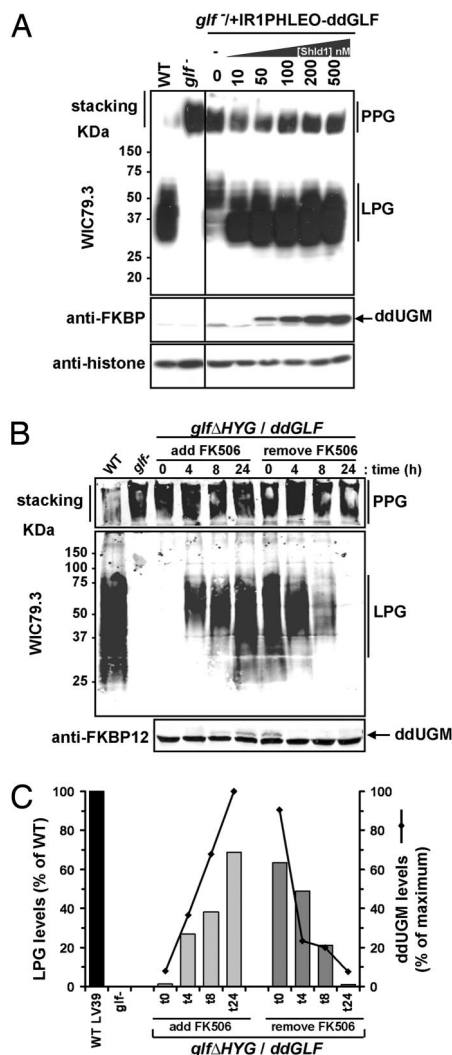


Fig. 5. Regulatable LPG and ddUGM expression in *L. major*. LPG, ddUGM, and histone 2A (loading control) levels were determined by Western blotting with appropriate antisera. Sample controls in all panels are WT *L. major* LV39c5 and *glf*^{-/-}, the homozygous *GLF* null mutant. (A) Analysis of a representative *glf*^{-/-} + *pIR1PHLEO-ddGLF* line. Parasites propagated in the absence of inducer were grown 24 h in the presence of Shld1 before harvest. (B) Analysis of a representative *ddGLF* knockin line. In lanes 3–6, FK506 (1 μM) was added to parasites grown without drug, and samples were harvested immediately (0) or at 4, 8, or 24 h thereafter. In lanes 7–10, parasites grown 24 h in the presence of FK506 (1 μM) were washed and suspended in fresh media lacking drug and were harvested immediately (0) or at 4, 8, or 24 h thereafter. (C) Quantitation of ddUGM (♦) and LPG (bars) levels seen in the experiment shown in B.

distinguishing WT and mutant phenotypes. However, expression levels from most common *Leishmania* vectors are considerably higher than those of chromosomal genes as a result of being multicopy episomes or from integration into the rRNA locus (31, 32). Thus, overexpression of a destabilized protein might leave protein levels high enough to fulfill normal function, perhaps accounting for the residual LPG expression evident in the studies above employing episomal ddUGM vectors. To test this, we generated a chromosomal knockin line expressing a single copy of ddUGM from the normal *GLF* locus.

We first created a generic knockin cassette that consists of a phleomycin resistance ORF (*PHLEO*) followed by a *Leishmania* intergenic region driving expression of the dd domain (Fig. S6A). This was then flanked on the 5' side with 915 bp of the 5' *GLF* flanking region and on the 3' side with the 1.5-kb *GLF* ORF (Fig.

S6B). This fragment was transfected into a heterozygous line bearing an *HYG* replacement of one *GLF* allele, followed by selection for *HYG* and *PHLEO* (Fig. 4). Several clones *glf*^{ΔHYG}/*ddGLF* were obtained with integration of the *PHLEO-ddGLF* cassette in the correct genomic locus (Fig. S6C), referred to here as *ddGLF* knockin lines.

Importantly, LPG levels were undetectable in the *ddGLF* knockin line cells grown in the absence of FK506 for 48 h (Fig. 5B, lane 3), in contrast to residual basal levels found in the *glf*^{-/-} + *pIR1PHLEO-ddGLF* transfectants (Fig. 5A, lane 3, and Fig. S6D). Western blotting revealed that in the presence of 1 μM FK506, ddUGM levels were ≈10-fold lower in the *ddGLF* knockin lines compared with the *glf*^{-/-} + *pIR1PHLEO-ddGLF* transfectants (Fig. S6D), as predicted. Thus, reduction in the levels of “destabilized” UGM by the knockin strategy resulted in improved down-regulation of LPG levels. Although some clonal variability was evident, as seen before, in 5 clones the LPG levels after induction were fully restored to WT levels (Fig. 5B, WT lane 1 vs. lane 6 or 7).

Kinetics of ddUGM-Dependent LPG Induction and Loss. Similar to many eukaryotic glycoconjugates, LPG synthesis occurs in the Golgi, followed by trafficking to the cell surface, where it is continuously “shed” with a half-life of about 3–7 h (33, 34). Thus, we examined the effects of regulated UGM expression on the kinetics of LPG acquisition and loss because this will affect its utility in functional studies of LPG. The kinetics of Shld1 stabilization of ddUGM appeared to be somewhat less rapid than seen with ddYFP reporter, with maximum ddUGM expression requiring 24 h of incubation with FK506. The appearance of LPG appeared to mirror ddUGM accumulation (Fig. 5C). The ddUGM levels dropped to near-basal levels 4 h after removal of FK506. In contrast, LPG levels fell more slowly, with a 3-fold decrease after 8 h, and complete loss of LPG was achieved 24 h after FK506 withdrawal (Fig. 5B and C). Thus, after ddUGM destabilization, LPG levels are lost from a cell more slowly than ddUGM, at rates similar to the rate of shedding established in previous studies.

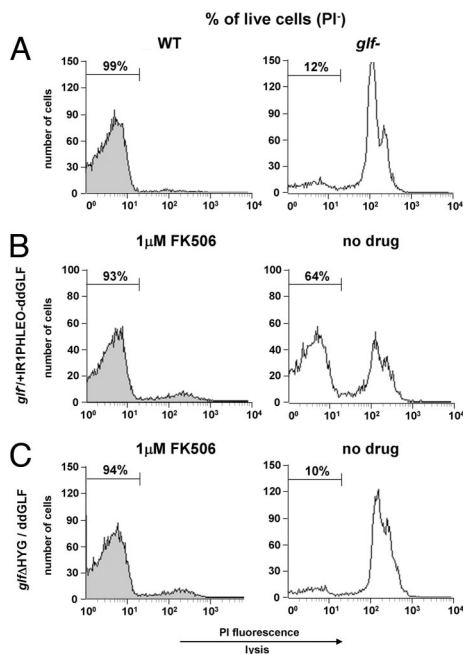
ddUGM-Dependent LPG Synthesis Recapitulates Expected Complement Sensitivities. The *Leishmania* surface is highly sensitive to perturbations involving loss and/or overexpression of many surface molecules, including LPG, gp46 and gp63, and the SHERP/HASP family, as judged by increased sensitivity to lysis by complement (19, 35–37). We used this assay to probe the faithfulness of the ddUGM-dependent LPG regulatory system to modulate the parasite surface. *glf*^{-/-} mutants were highly susceptible to lysis by complement (Fig. 6A), as seen in other LPG-deficient mutants (19).

The *glf*^{-/-} + *pIR1PHLEO-ddGLF* transfectants grown in the presence of FK506 were completely protected from lysis by complement (Fig. 6B), as expected given their strong LPG expression (Fig. 5A). However, in the absence of ligand most parasites (64%) remained complement-resistant, suggesting that the low LPG levels present in these cells were nonetheless sufficient to preclude lysis. This may reflect heterogeneity in the cellular population, most likely arising from variation in plasmid copy number.

Consistent with their high LPG levels, the knockin *ddGLF* parasites were highly resistant to complement in the presence of FK506 inducer. Importantly, in the absence of FK506 they became highly susceptible to complement lysis, similar to the *glf*^{-/-} control (Fig. 6C) and consistent with the absence of LPG (Fig. 5B). As noted earlier, clonal variability was evident, with many lines showing an intermediate phenotype.

Discussion

Here, we investigated the functionality of a recently developed system to regulate protein levels that consists of tagging the protein of interest with an FKBP dd, which in the absence of a stabilizing ligand (Shld1, rapamycin, or FK506) is targeted to



degradation by the proteasome (7). We show that this system performs as well as, and in some respects better than, that described in mammalian cells or Apicomplexan parasites (8, 9). The FKBP/Shld1 system functioned well in 2 *Leishmania* species representing different subgenera, and it conferred strong ligand-dependent expression for all 4 parasite proteins tested (Fig. 1 and Fig. S3). The kinetics of induction and degradation were also favorable, with full induction occurring within 8 h and loss occurring within 2 h of drug removal (Fig. 3 and Table S1). Lastly, the utility of the system was shown by its ability to regulate expression of the important surface glycoconjugate LPG (Fig. 5).

Hence, the ddFKBP system constitutes a promising molecular tool for the regulation of protein levels in *L. major*, with relatively fast effects on protein levels upon addition and removal of rapamycin analogs. A second advantage arises from the property termed “tunability,” in that the level of expression can be adjusted quantitatively and homogeneously across the cell population by titration of varying amounts of ligand (Fig. S14) (7).

Rapamycin and FK506 Are Cost-Effective Ligands for the dd-Inducible System in *Leishmania*. Although Shld1 is nontoxic to cells from many species tested in vitro thus far, and to mice as well, it is relatively expensive and of limited availability. Shld1 was 10-fold more potent in stabilization of the ddYFP fusion protein in *L. major* than reported for mammalian cells (Fig. 2B and Table S1) (7). Although alternate FKBP ligands, such as rapamycin and FK506, are relatively inexpensive and available, in many species they show toxicity due to inhibition of critical signaling pathways involving calcineurin and TOR kinases (38–40). However, *Leishmania* were relatively insensitive to FK506 and rapamycin (IC₅₀: 7.7 and 4.9 μM), and full induction of ddYFP or ddLUC expression was achieved by 1 μM (Figs. 1 and 2 and Table S1).

The potent, efficacious properties of FK506 and rapamycin with the ddFKBP system in *Leishmania*, in combination with their relative lack of toxicity in vitro, establish these as attractive alter-

natives to the more costly Shld1. Potentially, these ligands may be used in other organisms, depending on the relative potency of ddFKBP induction vs. toxicity. Although FK506 is highly toxic to mammalian cells, rapamycin has been used productively in mice in vivo (41). However, it seems likely that their immunosuppressive properties may prove problematic because of the complex interactions of *Leishmania* parasites and their host immune system (42).

An Inducible System for LPG Expression. Expression of a ddUGM construct within the *glf⁻* mutant resulted in low levels of LPG in the absence of ligand, but full LPG expression upon induction (Fig. 5). Robust LPG expression was induced at the lowest concentration of Shd1 tested (10 nM), at least 20-fold lower than required to get maximal ddUGM expression (Fig. 5A). Remarkably, the low residual levels of LPG expression in the uninduced *glf⁻/+pIR1PHLEO-ddGLF* line were sufficient to confer considerable resistance to lysis by complement, a sensitive, functional readout. Thus, we tested an alternative strategy, generating a ddGLF knockin at the normal *GLF* locus. As expected, the ddGLF knockins showed 10-fold lower levels of ddUGM expression in the presence of ligand compared with the *ddGLF* episomal complemented line. Importantly, these showed undetectable ddUGM and LPG levels in the absence of ligand and were completely susceptible to complement lysis. In contrast, in the presence of ligand, LPG levels and complement resistance closely resembled that of WT parasites (Figs. 5 and 6). Together, these data confirm the biochemical and phenotypic “faithfulness” of the inducible system in its on and off states.

We anticipate that this inducible system will prove valuable in further studies of the diverse roles of LPG in survival of the *Leishmania* parasite. These studies will need to take into account the kinetic properties of both ddUGM-dependent LPG synthesis and LPG shedding described here. LPG-dependent *Leishmania* interactions with its mammalian host take place rapidly after deposition by the sand fly vector, within a few minutes (complement lysis, macrophage uptake) to hours (inhibition of phagolysosomal fusion, differentiation), and are completed within 24 h, when the parasites have successfully established infection as amastigotes, which lack LPG (15, 43). The kinetics of ddUGM-dependent LPG appearance and loss, requiring upwards of 4 h for the significant gain or loss, may render its use in these settings problematic. Nonetheless, the tunability of the ddFKBP system would permit studies of the quantitative dependency of LPG levels under these circumstances. In contrast, the role of LPG in the sand fly vector should be amenable to study by the ddUGM system, because these LPG-dependent interactions span days, including initial survival within the peritrophic matrix-enclosed blood meal, binding of procyclics to the parasite midgut lectin PpGalec, and release as infective metacyclics (43).

We anticipate more generally that the properties of the ddFKBP regulatory system described here will be applicable to the study of many other parasite proteins in a variety of circumstances relevant to the parasite infectious cycle. Our studies identify several important variables to consider in the design of such strategies. First, as seen in other systems, there can be significant variation among clonal lines, the origins of which are not always evident, suggesting that screening at both the biochemical and biological levels for lines behaving properly will be required. Second, it remains to be determined whether the destabilization module will function effectively in cellular compartments other than the cytoplasm; for example, the secretory pathway or organelles, such as the glycosome or mitochondrion. Lastly, it is clear that genetic strategies that minimize the level of expression of the protein in the uninduced state—for example, using knockin rather than episomal or high-level expression vectors—may have the highest probability of crossing biologically relevant thresholds in the off state.

Materials and Methods

FKBP dd Ligands. Shld1 was generously provided by Thomas Wandless (Stanford University, Stanford, CA). Rapamycin and FK506 were purchased from LC Laboratories.

Parasites. *L. major* Friedlin clone V1 (MHOM/IL/81/Friedlin) and LV39 clone 5 (Rho/SU/59/P) were grown in M199-based medium (31). *L. braziliensis* (MHOM/BR/75/M2903) was grown in Schneider's Insect Medium (Sigma-Aldrich) supplemented with 10% heat-inactivated FBS, 2 mM L-glutamine, 500 units of penicillin, and 50 μ g/mL streptomycin. Metacyclics were isolated by negative selection using peanut-agglutinin (PNA) (44).

Unless otherwise indicated, all experiments were performed with promastigotes during early to mid log phase of exponential growth (typically $<8 \times 10^6$ parasites per milliliter) because some proteins are extensively degraded in stationary phase because of the activation of a lysosomal pathway (45). These include YFP and GFP but not DsRed2 or Luciferase.

DNA Constructs and Genetic Manipulation of Parasites. The description of the molecular constructs used in this work is presented in Figs. S5C and S6A, Table S2, and Table S3. DNAs were introduced directly or after digestion with appropriate restriction enzymes, followed by dephosphorylation. Transfection was performed by using high-voltage electroporation (46), and clonal transfectants were recovered after plating on semisolid media containing 1% agar, which contained the appropriate concentrations of selective drugs.

Flow Cytometry, Luciferase Assay, and Western Blotting. YFP expression was determined by flow cytometry using a FACSCalibur cytometer (Becton

Dickinson) and quantitated by using CellQuest software (Becton-Dickinson). Luciferase activity was determined by suspending 10^6 cells in 200 μ L of M199 medium in a 96-well plate containing 1 μ L of luciferin (30 μ g/mL). After 10 min of incubation, the plate was imaged by using a Xenogen IVIS Photoimager, and luciferase activity was quantified as photons per second (p/s). Preparation of samples for Western blotting and the antisera used are described in SI Text.

Complement Lysis Assay. Lysis by complement was assayed as described previously (19). Briefly, late log-phase promastigotes were washed once in DMEM, and 1×10^6 washed cells were then incubated at room temperature for 30 min with 4% human serum in DMEM containing 0.4 μ g/mL propidium iodide (PI), followed by flow cytometry.

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